

*Citation for published version:*

O'Keefe, KM, Wilk, MM, Leech, JM, Murphy, AG, Laabei, M, Monk, IR, Massey, RC, Lindsay, JA, Foster, TJ, Geoghegan, JA & McLoughlin, RM 2015, 'Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* bloodstream infection', *Infection and immunity*, vol. 83, no. 9, pp. 3445-3457.  
<https://doi.org/10.1128/IAI.00358-15>

*DOI:*

[10.1128/IAI.00358-15](https://doi.org/10.1128/IAI.00358-15)

*Publication date:*

2015

*Document Version*

Publisher's PDF, also known as Version of record

[Link to publication](#)

## University of Bath

### Alternative formats

If you require this document in an alternative format, please contact:  
[openaccess@bath.ac.uk](mailto:openaccess@bath.ac.uk)

#### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

#### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1   **Manipulation of autophagy in phagocytes facilitates *Staphylococcus***  
2   ***aureus* bloodstream infection.**

3

4   Kate M. O'Keeffe<sup>1</sup>, Mieszko M. Wilk<sup>1</sup>, John M. Leech<sup>1</sup>, Alison G. Murphy<sup>1</sup>,  
5   Maisem Laabei<sup>2</sup>, Ian R. Monk<sup>4</sup>, Ruth C. Massey<sup>2</sup>, Jodi A. Lindsay<sup>3</sup>, Timothy J.  
6   Foster<sup>4</sup>, Joan A. Geoghegan<sup>4</sup> and Rachel M. McLoughlin<sup>1#</sup>

7   <sup>1</sup>Host Pathogen Interactions Group, School of Biochemistry and Immunology,  
8   Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland

9   <sup>2</sup>Department of Biology and Biochemistry, University of Bath, Claverton Down,  
10   Bath, UK

11   <sup>3</sup>Institute for Infection and Immunity, St George's, University of London,  
12   London, UK

13   <sup>4</sup>Microbiology Department, Moyne Institute of Preventative Medicine, Trinity  
14   College Dublin, Dublin 2, Ireland

15

16

17   Running title: *S. aureus* and autophagy

18

19   <sup>#</sup>To whom correspondence should be addressed: rachel.mcloughlin@tcd.ie

20

21

22

23

24 **ABSTRACT**

25 The capacity for intracellular survival within phagocytes is likely a critical  
26 factor facilitating *S. aureus* dissemination in the host. To date, the majority of  
27 work on *S. aureus*-phagocyte interactions has focused on neutrophils and to a  
28 lesser extent macrophages, yet we understand little about the role played by  
29 dendritic cells (DCs) in the direct killing of this bacterium. Using bone-marrow-  
30 derived DCs (BMDCs) we demonstrate for the first time that DCs can  
31 effectively kill *S. aureus*, however certain strains of *S. aureus* have the  
32 capacity to evade DC (and macrophage) killing by manipulation of autophagic  
33 pathways. Strains with high levels of Agr activity were capable of causing  
34 autophagosome accumulation, were not killed by BMDCs and subsequently  
35 escaped from the phagocyte, exerting significant cytotoxic effects.  
36 Conversely, strains that exhibited low levels of Agr activity failed to  
37 accumulate autophagosomes and were killed by BMDCs. Inhibition of the  
38 autophagic pathway by treatment with 3-Methyladenine restored the  
39 bactericidal effects of BMDCs. Using an *in vivo* model of systemic infection we  
40 demonstrated that the ability of *S. aureus* strains to evade phagocytic cell  
41 killing and to survive temporarily within phagocytes correlated with  
42 persistence in the periphery and that this effect is critically Agr dependent.  
43 Taken together our data suggests that strains of *S. aureus* exhibiting high  
44 levels of Agr activity are capable of blocking autophagic flux, leading to the  
45 accumulation of autophagosomes. Within these autophagosomes the bacteria  
46 are protected from phagocytic killing, thus providing an intracellular survival  
47 niche within professional phagocytes, which ultimately facilitates  
48 dissemination.

49

## 50 INTRODUCTION

51 *Staphylococcus aureus* causes a wide range of pathologies from superficial  
52 skin infections to more serious invasive infections associated with significant  
53 morbidity and mortality. In severe cases, localized infections can lead to  
54 bacterial invasion of the vascular system causing life-threatening conditions  
55 such as bacteremia and sepsis. A key factor facilitating this dissemination is  
56 the impressive arsenal of immune evasion strategies available to *S. aureus*  
57 that enables it to evade recognition and killing by the host immune system (1).  
58 Identifying and disarming the mechanisms by which this organism  
59 circumvents the host's immune system is an important strategy for identifying  
60 novel therapies.

61

62 Although classically considered an extracellular bacterium, *S. aureus* is  
63 capable of invading and persisting within a variety of non-professional  
64 phagocytic host cells (2) facilitating tissue persistence and relapsing disease.  
65 Strikingly, this organism is also capable of manipulating professional  
66 phagocytes and there is evidence that *S. aureus* can survive within  
67 monocytes, macrophages and even neutrophils (3-7). Unlike resident tissue  
68 cells, professional phagocytes are mobile and represent an opportunity for the  
69 bacterium to disseminate from the primary focus of infection to systemic sites.  
70 In a similar mechanism to that employed by traditional intracellular bacteria  
71 such as *Mycobacterium tuberculosis* and *Listeria monocytogenes* that utilize  
72 monocytes to disseminate via the bloodstream (8, 9), it has been proposed  
73 that *S. aureus* may be capable of subverting neutrophils to facilitate its  
74 dissemination (10). *S. aureus* has also been shown to persist within human

75 monocyte-derived macrophages (7) suggesting that these cells may also  
76 provide a potential intracellular niche to facilitate *S. aureus* dissemination *in*  
77 *vivo*. The bulk of the research conducted into the survival within or killing of *S.*  
78 *aureus* by phagocytes has focused on neutrophils and, to a lesser extent  
79 macrophages. To date, the contribution of dendritic cells to direct killing of *S.*  
80 *aureus*, and the capacity of *S. aureus* to manipulate these particular  
81 phagocytes has not been explored.

82

83 Despite the fact that the environment inside phagocytes is less than  
84 hospitable, gaining an intracellular niche, even briefly, within these cells  
85 affords a window of opportunity for extended survival and potential  
86 dissemination. Critical to survival is the ability to avoid destruction within  
87 phagolysosomes and *S. aureus* is equipped with a number of strategies to  
88 resist phagolysosomal killing (11-13). Having circumvented these killing  
89 mechanisms the bacterium can then escape into the cytoplasm, which in most  
90 cases, eventually leads to host cell death, releasing the bacteria into the  
91 extracellular space where it has the opportunity to replicate and infect other  
92 host cells. Phagosomal escape by *S. aureus* has been shown to depend upon  
93 the regulatory system encoded by the *agr* locus (7, 14, 15) which controls  
94 expression of a number of virulence factors including the secreted toxin  $\alpha$ -  
95 haemolysin (Hla), a critical effector molecule essential for *S. aureus* survival  
96 within macrophages (7). Phenol-soluble modulins (PSMs) are small cytotoxic  
97 alpha-helical peptides. They are categorized into two classes, PSM $\alpha$  and  
98 PSM $\beta$  peptides. PSM $\alpha$  peptides are regulated by the Agr system and enable  
99 phagosomal escape by *S. aureus* from both non-professional (16) and

100 professional phagocytes (17, 18). Survival within neutrophils appears to be  
101 dependent upon the accessory regulator SarA, which facilitates the survival of  
102 *S. aureus* within large vacuoles that are not competent for fusion with  
103 lysosomes (5). While it is clear that phagocytes are critically important for  
104 effective clearance of *S. aureus* during an infection, it may be that the  
105 intracellular locale of the bacterium post-phagocytosis will dictate whether or  
106 not the phagocytes contribute to host protection or inadvertently play a  
107 deleterious role.

108

109 Autophagy is an important homeostatic process in eukaryotic cells critical for  
110 cell survival. Damaged cytosolic components are removed and recycled in  
111 double-membrane vacuoles called autophagosomes that are characterized by  
112 the recruitment of microtubule-associated protein 1 light chain 3 (LC3)  
113 conjugated to phosphatidylethanolamine (LC3-II) to its membrane (19). These  
114 autophagosomes then fuse with lysosomes and are digested. This process of  
115 autophagosome formation and eventual degradation is termed autophagic flux  
116 (20). Autophagy also plays an important role in host defense against bacteria  
117 that can invade host cells such as *Streptococcus pyogenes* (21) or facultative  
118 intracellular pathogens such as *Mycobacterium tuberculosis* (22). These  
119 organisms are sequestered in autophagosomes, which then deliver the  
120 bacteria to the lysosomes for destruction. Some microorganisms (e.g. *Coxiella*  
121 *burnetti* and *Porphyromonas gingivalis*) have evolved mechanisms to subvert  
122 the autophagic machinery of the cell, delaying autophagosomal maturation  
123 and lysosomal fusion thus creating a survival niche within autophagosomes  
124 (23). *S. aureus* can localize to autophagosomes and inhibit lysosomal fusion

125 within HeLa cells while proliferation of *S. aureus* was impaired within  
126 fibroblasts deficient in the autophagy protein Atg5 (24), indicating an essential  
127 role for the autophagy pathway in facilitating intracellular survival of *S. aureus*  
128 within non-professional phagocytic cells. In this study, a strain that expresses  
129 low levels of *agr* failed to colocalise with autophagosomal markers identifying  
130 the requirement for Agr-regulated genes to engage autophagosomes.

131

132 Whether or not *S. aureus* can manipulate the autophagic process in  
133 professional phagocytes as a means to evade killing remains to be  
134 established. We hypothesized that subversion of autophagy in professional  
135 phagocytes could provide *S. aureus* with a means to preserve a temporary  
136 intracellular survival niche, in order to facilitate dissemination. We  
137 demonstrate the strain-dependent ability of *S. aureus* to induce accumulation  
138 of autophagosomes in phagocytes, which appears to correlate with inter-strain  
139 differences in Agr expression. Strains with high levels of Agr activity became  
140 associated with autophagosomes, were not killed by phagocytic cells *in vitro*  
141 and demonstrated extended intracellular survival within phagocytes *in vivo*.

## 142 MATERIALS AND METHODS

### 143 Bacterial strains

144 *S. aureus* strains SH1000 (Clonal complex (CC) 8) and PS80 (CC30) have  
145 been previously described (25, 26). *S. aureus* clinical isolates were from blood  
146 culture bottles of patients diagnosed with *S. aureus* bacteremia at St George's  
147 Healthcare NHS Trust, London. Two isolates were used repeatedly  
148 throughout this study; Sa68 and Sa279. Both of these strains are methicillin  
149 sensitive and belong to the lineage CC1.

150

151 The expression of enhanced green fluorescent protein (GFP) (27) in the PS80  
152 background was achieved through the integration of a non-replicative  
153 integrase vector (pIMC11-GFP) into the phage 11 attachment site. Expression  
154 of eGFP is under the control of the  $P_{xyl/tetO}$  promoter, without repression from  
155 TetR. Chromosomal integration of PS80::pIMC11-GFP was validated with  
156 oligonucleotides IM293/IM294, which amplify across the site of integration  
157 yielding a 0.7kb product in PS80 and a 3.4kb product in PS80::pIMC11-GFP.

158

159 Deletion of the *agr* locus (*agrBDCA* genes) within PS80 was achieved by  
160 allelic exchange using pIMAY (28). Primers *agr1* and *agr2* amplified 532 bp of  
161 DNA upstream of *agrB* and primers *agr3* and *agr4* amplified 535 bp of DNA  
162 located downstream of the *agrA* gene (Table 1). The PCR products were  
163 denatured and allowed to reanneal via the complementary sequences in  
164 primers *agr2* and *agr3*. This was used as template for PCR using primers  
165 *agr1* and *agr4*. The amplicon was cloned into pIMAY (28) between *Sall* and  
166 *EcoRI* restriction sites using sequence and ligase independent cloning (29)



167 and the resulting plasmid (pIMAY:: $\Delta agr$ ) was transformed into DC10B and  
168 verified by DNA sequencing. The plasmid was transformed into  
169 electrocompetent PS80 and deletion of the *agr* genes was achieved by allelic  
170 exchange as previously described (28). The deletion was confirmed by DNA  
171 sequencing of a PCR amplicon generated using PS80 $\Delta agr$  genomic DNA as  
172 template and the primers *agr* OUT F and *agr* OUT R. The mutant did not  
173 produce delta haemolysin on sheep blood agar.

174

175 All bacteria were cultivated from frozen stocks for 24 hours at 37°C on agar  
176 plates. Bacterial suspensions were then prepared in PBS and the  
177 concentrations estimated by measuring the absorbance of the suspension  
178 read at 600nm. CFUs were determined by plating serial dilutions of each  
179 inoculum.

180

181 In the case of PS80-GFP, log phase growth was required for optimal GFP  
182 expression. A single colony was inoculated into TSB overnight and a  
183 subculture to fresh TSB taken the following morning. The concentration of  
184 bacteria in the broth was determined by measuring absorbance at 600nm and  
185 confirmed by streaking on agar plates.

186

187 For immunofluorescent analysis, bacteria were stained with Cell Trace Violet  
188 (CTV, Life Technologies). Stationary phase bacteria in PBS at the appropriate  
189 OD were incubated with CTV for 20min at 37°C under rotation. They were  
190 then washed and resuspended in PBS prior to infection of cells.

191

192 **Animals**

193 Groups of wild type C57BL/6 mice (6-8 weeks) were housed under specific  
194 pathogen free (SPF) conditions in the Trinity College Dublin Comparative  
195 Medicines facility. All animal experiments were conducted in accordance with  
196 the recommendations and guidelines of the Health Products Regulatory  
197 Authority (HPRA), the competent authority in Ireland, and in accordance with  
198 protocols approved by Trinity College Animal Research Ethics Committee.

199

200 **Cell Culture**

201 Bone marrow derived dendritic cells (BMDCs) were prepared by culturing  
202 bone marrow cells isolated from C57BL/6 mice with granulocyte-macrophage  
203 colony stimulating factor (GM-CSF) as described previously (30). On day 10,  
204 loosely adherent cells were collected, washed and reseeded at a  
205 concentration of  $2 \times 10^5$  cells/well in media without antibiotic, and rested  
206 overnight.

207

208 Peritoneal macrophages were isolated as previously described (31) and  
209 seeded at  $2 \times 10^5$  cells/well in media containing no antibiotics.

210

211 Immortalized Bone Marrow derived Macrophages (iBMM) stably expressing  
212 EGFP-LC3 (GFP-LC3) (32) were cultured in cRPMI (complete Roswell Park  
213 Memorial Institute) media under constant selection with  $10 \mu\text{g/ml}$  puromycin.  
214 Cells were seeded at  $1 \times 10^6$  cells/well on poly-L-lysine coated 19mm  
215 coverslips in 12 well plates.

216

217 **Infection of phagocytes**

218 Cells were infected with live *S. aureus* at multiplicities of infection (MOI) of 10  
219 or 100 for the indicated times. In some cases, prior to infection cells were  
220 incubated with 10mM 3-Methyladenine (3-MA, Sigma) for 30min. At 2 hours  
221 post infection media was replaced with fresh media containing gentamicin  
222 (200 µg/ml) for 1 hour to kill extracellular bacteria. This media was replaced  
223 with fresh media containing no antibiotics and this was considered time 0.

224

225 For assessment of total killing, cells were infected with live *S. aureus* at MOI  
226 10 or 100 for the indicated time points and were not gentamicin treated.

227

228 **Assessment of bacterial killing**

229 At the indicated time point, infected cells were spun down, the supernatant  
230 removed and cells lysed by the addition of 20 µl 0.1% Triton-X 100. The  
231 supernatant was then re-introduced into the well and mixed with the cell  
232 lysate. Serial dilutions of the suspension were prepared in PBS and plated on  
233 TSA to determine the CFU/ml. Bacterial killing was determined as the %  
234 reduction of CFU in wells containing bacteria and phagocytes as compared to  
235 wells containing bacteria only.

236

237 **Assessment of bacterial escape**

238 *S. aureus* infected BMDCs underwent gentamicin treatment as described  
239 above. At specific time points the cell free supernatants were collected,  
240 serially diluted in PBS and plated on TSA to determine the number of bacteria

241 that had escaped into the media, measured as the fold increase in Log  
242 CFU/well from time 0.

243

#### 244 **Cell viability assays**

245 To assess *S. aureus* induced cytotoxicity, BMDCs were infected and treated  
246 with gentamicin as described above. LDH release was measured using the  
247 Pierce LDH Cytotoxicity Assay kit (Thermo Scientific) according to the  
248 manufacturer's instructions. In some cases cell viability was assessed by the  
249 addition of Propidium Iodine (PI, 1 µg/ml (eBioscience)) and analysis by flow  
250 cytometry.

251

#### 252 **Vesicle Lysis Test**

253 Phospholipid vesicles were prepared as described previously (33). Vesicle  
254 lysis test (VLT) was performed using a 1:1 ratio of bacterial supernatant  
255 (cultures grown for 18 h) and pure vesicles and fluorescence intensity  
256 measured at excitation and emission wavelengths of 485-520 nm respectively  
257 on a FLUOstar fluorometer (BMG labtech). Positive and negative controls  
258 were pure vesicles with 0.01% Triton X-100 and HEPES buffer, respectively.

259

#### 260 **Measurement of RNA III expression by qRT-PCR**

261 *S. aureus* RNA was isolated using the RNeasy Mini Kit (Qiagen) according to  
262 the manufacturer's instructions with the addition of turbo DNase (Ambion)  
263 following the purification step. RNA was quantified using RNA BR kit (Qubit)  
264 and reverse transcription was performed using the ProtoScript Taq RT-PCR  
265 kit (New England Biolabs) according to manufacturer's instructions using

266 random primers. Standard curves were generated for both gyrase B [*gyrFW*:  
267 5'-CCAGGTAAATTAGCCGATTGC-3'; *gyrRV*: 5'AAATCGCCTGCGTTCTAGA  
268 G] and RNAlII primers [*rnalIIFW*: 5'- GAAGGAGTGATTTCAATGGCACAAG-  
269 3'; *rnalIIRV*: 5' GAAAGTAATTAATTATTCATCTTATTTTTTAGTG AATTG-3']  
270 using genomic DNA to determine primer efficiency. Real-time PCR was  
271 performed using the SYBR green PCR master mix (Applied Biosystems) as  
272 previously described (33).

273

#### 274 **Western immunoblotting**

275 To detect LC3, BMDCs were infected and treated with gentamicin as  
276 described above. At specified time points BMDCs were lysed in NP-40 lysis  
277 buffer. The protein concentration of the lysates was measured using a  
278 Bradford assay (Thermo Scientific) and equal concentrations of protein were  
279 loaded to each lane of the gel. Samples were separated on a 15% SDS-  
280 polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF)  
281 membrane. The membrane was blocked with 5% (w/v) milk before being  
282 probed with antibody (rabbit anti-LC3 - 1/1000 (Cell Signaling), HRP-  
283 conjugated goat anti-rabbit immunoglobulin G (IgG) - 1/10000 (Jackson  
284 Immune)). The membrane was developed with ECL (Mybio) on Bio-Rad  
285 GelDoc.

286 To detect Hla expression, proteins from filtered bacterial supernatant were  
287 concentrated by trichloroacetic acid precipitation, separated on a 12.5% SDS-  
288 polyacrylamide gel and transferred to PVDF. The membrane was blocked  
289 in 10% (w/v) milk and probed with polyclonal rabbit anti-Hla IgG (1:1000, (34))  
290 followed by HRP-conjugated protein A (Sigma). Reactive bands were

291 visualized using the LumiGLO reagent and peroxide detection system (Cell  
292 Signaling Technology).

293

#### 294 **Confocal imaging**

295 BMDCs were infected, gentamicin treated as described above and  
296 Monodansylcadaverine (MDC, 50 $\mu$ M) added 15 minutes prior to cell fixation.  
297 Cells were then fixed in 2% paraformaldehyde (PFA (Thermo Scientific)).  
298 Alternatively, GFP+LC3-BMMs were infected and treated with gentamicin as  
299 described above. At specific time points post-infection, cells were fixed in 2%  
300 PFA and permeabilised in Triton-X100 (0.1% in PBS). Non-specific binding  
301 was blocked by incubation in 5% BSA before cells were incubated with Alexa  
302 Fluor 555 - conjugated phalloidin (1/100 (Life Technologies)) for 1 hour to  
303 stain actin.

304 The coverslips were mounted onto glass slides with fluorescent Mounting  
305 medium (DakoCytomation) and analyzed on an Olympus FV1000 laser  
306 scanning confocal microscope.

307

#### 308 ***In vivo* intraperitoneal infection model**

309 Mice were infected with *S. aureus* (5 x 10<sup>8</sup> CFU) via intraperitoneal (i.p.)  
310 injection. At specific time points post-infection peritoneal exudate cells (PEC)  
311 were isolated by lavage of the peritoneal cavity with sterile PBS. Lavage fluid  
312 was serially diluted in PBS and plated on TSA to determine the bacterial  
313 burden at the site of infection. Spleens were isolated and homogenized in 2ml  
314 of sterile PBS. Tissue homogenates were then serially diluted in PBS and  
315 plated on TSA to determine the tissue bacterial burden. Blood was collected

316 by cardiac puncture with a 27-gauge needle and a heparinized 1ml syringe.  
317 The CFU/ml of blood was determined by serial dilution and plating on TSA  
318 plates.

319

320 To isolate leukocytes, blood was layered onto Histopaque-1083 (Sigma) for  
321 density gradient centrifugation. Leukocytes were collected between the  
322 plasma layer and the pellet containing red blood cells (RBCs) and  
323 extracellular bacteria (35). Isolated leukocytes were then washed well and  
324 resuspended in Fcy block for flow cytometric analysis or lysed in sterile water  
325 to quantify cell-associated CFU.

326

#### 327 **Flow cytometry**

328 PEC or blood leukocytes were blocked in Fcy block (1 $\mu$ g/ml, eBioscience)  
329 then surface-stained with fluorochrome-conjugated antibodies against Ly6G  
330 (clone 1A8, BDBioscience), F4/80 (clone BM8, eBioscience), CD11c (clone  
331 N418, eBioscience) and CD11b (clone M1/70, eBioscience). Flow cytometric  
332 data were acquired with a BD FACSCanto II (BD Biosciences) and analyzed  
333 using FlowJo software (Tree Star).

334

335 To assess the rate of *S. aureus* phagocytosis by BMDCs, cells that had been  
336 infected with CTV-labeled *S. aureus* for 30 minutes or 2 hours, were  
337 incubated with gentamicin (200 $\mu$ g/ml) for 1 hour, washed and fixed in 2%  
338 PFA. They were then analyzed on BD FACSCanto II by gating on Forward-  
339 Scatter and Side-Scatter and % CTV+ cells assessed.

340

341 **Statistical analysis**

342 Statistical analysis was carried out using GraphPad Prism statistical analysis  
343 software. Differences between groups were analyzed by the unpaired  
344 Students t test or analysis of variance (ANOVA) with appropriate post-test and  
345 using repeated measurers where required.  $P < 0.05$  was considered  
346 statistically significant.

347



348 **RESULTS**

349 **Killing of *S. aureus* by dendritic cells and macrophages is strain**  
350 **dependent.**

351 Despite the fact that dendritic cells have been shown to be involved in  
352 coordinating the immune response to *S. aureus* infection, their contribution to  
353 direct bacterial killing remains to be fully established (36, 37). We compared  
354 the bactericidal capacities of these phagocytic cells to that of macrophages  
355 which have a more clearly defined role in direct killing of *S. aureus* (38).  
356 Primary BMDCs were infected with two laboratory strains of *S. aureus* at an  
357 MOI 10 (Figure 1A) and MOI 100 (Figure 1B) and bacterial killing was  
358 monitored over time. Within 6 hours of infection, approximately 70% of  
359 SH1000 was killed and by 16 hours almost 100% of SH1000 had been killed  
360 by the BMDCs at either MOI. In contrast, the BMDCs were unable to kill *S.*  
361 *aureus* strain PS80.

362

363 Interestingly, the ability of BMDCs to kill SH1000 appeared to be MOI-  
364 dependent. It was reported previously that BMDCs were unable to kill SH1000  
365 at an MOI 0.1 (36). We also failed to detect any killing of SH1000 by BMDCs  
366 at this low MOI but the ability of BMDCs to kill SH1000 by 16 hours became  
367 apparent at as low an MOI as 2 (97.7±1.7% killing).

368

369 To establish if the inability to kill *S. aureus* strain PS80 was specific to  
370 dendritic cells, we infected primary peritoneal macrophages with both strains  
371 of *S. aureus* at MOI 100. Similar to that observed with the BMDCs, peritoneal  
372 macrophages efficiently killed SH1000 but were unable to kill PS80 (Figure

373 1C). Interestingly, in our hands BMDCs and macrophages demonstrated a  
374 similar capacity to kill *S. aureus* strain SH1000 (the % killing at 16 hours was  
375  $90 \pm 6.8\%$  in BMDCs compared to  $78.3 \pm 6.6\%$  in macrophages). Taken  
376 together these results suggest that BMDCs are capable of killing *S. aureus*  
377 but that strain-dependent differences may impact upon the ability of both  
378 macrophages and BMDCs to kill the bacterium.

379

380 ***S. aureus* strain PS80 but not SH1000 can escape from dendritic cells**  
381 **causing associated cytotoxicity.**

382 Given that BMDCs had a different capacity to kill *S. aureus* strains PS80 and  
383 SH1000, we wanted to confirm that both strains were phagocytosed by  
384 BMDCs at the same rate. BMDCs were infected with CTV-labeled *S. aureus*  
385 at MOI 100, and the uptake of bacteria into the BMDCs assessed after 30  
386 minutes and 2 hours, following gentamicin treatment to kill any bacteria that  
387 had not been phagocytosed. At 30 min post infection PS80 and SH1000 were  
388 phagocytosed by BMDCs to the same extent, with ~30% of BMDCs staining  
389 positively for CTV-labeled PS80 or SH1000 (Figure 2A). At 2 hours post  
390 infection the % of cells that were CTV-PS80+ increased, alluding to the  
391 survival of this strain inside the cells.

392

393 *S. aureus* strains SH1000 and PS80 were both phagocytosed by BMDCs to  
394 the same extent but following phagocytosis PS80 was not killed. To establish  
395 whether PS80 escaped from the BMDCs, cells were allowed to phagocytose  
396 the bacteria and any extracellular bacteria were killed by the addition of the  
397 bactericidal antibiotic gentamicin. Cells were washed and incubated in fresh

398 medium and the escape of viable bacteria into the supernatant was measured  
399 after 6 and 12 hours incubation. By 6 hours there was evidence of PS80 but  
400 not SH1000 escaping from the BMDCs. By 12 hours the level of PS80 in the  
401 cell culture supernatant significantly higher than SH1000 (Figure 2B). Similar  
402 results were obtained following infection with MOI 10 (data not shown).

403

404 To establish if the escape of *S. aureus* from BMDCs was associated with  
405 cytotoxicity, LDH release from the infected BMDCs was measured. LDH  
406 activity was similar in uninfected BMDCs and BMDCs infected with SH1000 at  
407 both 6 h and 12 h post-infection, indicating that SH1000 had no effect on the  
408 viability of the infected cells. In contrast, BMDCs infected with PS80 had  
409 significantly higher levels of LDH in the supernatant compared to cells  
410 infected with SH1000 or uninfected BMDCs at both time points (Figure 2C),  
411 indicating significant cytotoxicity.

412

413 **Identification of *S. aureus* bloodstream infection isolates with the ability**  
414 **to escape phagocytic killing.**

415 *S. aureus* PS80 and SH1000 are both well-characterized laboratory strains.  
416 However, their relevance to clinical isolates may be limited. Accordingly,  
417 isolates that were recovered from *S. aureus* bacteremia patients were  
418 collected and screened for cytotoxic effects. BMDCs were infected with each  
419 isolate at MOI 100 and the viability of the infected DCs was assessed after 24  
420 hours by staining with PI. The clinical isolates clustered together into one  
421 group that was cytotoxic to BMDCs in a similar manner to PS80, a second

422 group that did not exert any cytotoxic effects, akin to SH1000 and a third,  
423 intermediate group (Figure 3A).

424

425 A representative isolate from both the “PS80-like” group and the “SH1000-  
426 like” group were selected for analysis, *S. aureus* 68 (Sa68) and *S. aureus* 279  
427 (Sa279). BMDCs were infected with Sa68 or Sa279 at MOI 100. The BMDCs  
428 were capable of killing strain Sa279 but were unable to kill strain Sa68 (Figure  
429 3B). This data suggests that Sa68 is similar to PS80 and may be capable of  
430 escaping from phagocytes. We confirmed that both strains were  
431 phagocytosed by BMDCs at a similar rate by CTV-labeling the bacteria and  
432 infecting BMDCs as described above. Similar to the uptake of PS80 and  
433 SH1000, approximately 30% of BMDCs were associated with CTV+ Sa279 or  
434 Sa68 by 30 min post infection (Figure 3C). We then assessed the ability of  
435 Sa68 to escape from the BMDCs. After 12 hours the level of Sa68 in the cell  
436 culture supernatant was significantly higher than Sa279 (Figure 3D).

437

438 To establish if the ability of Sa68 to escape from the BMDCs correlated with  
439 cytotoxicity, cells were infected with Sa68, Sa279 or left uninfected. Following  
440 gentamicin killing of extracellular non-phagocytosed bacteria, the LDH release  
441 was monitored at 6 h and 12 h. The level of cytotoxicity (LDH release)  
442 associated with Sa68-infected cells was significantly higher than that of  
443 Sa279-infected cells or the uninfected control cells (Figure 3E).

444

445 **Infection with PS80, but not SH1000, was associated with increased**  
446 **accumulation of LC3-II+ autophagosomes.**

447 *S. aureus* has previously been shown to associate with autophagosomes in  
448 non-professional phagocytic cells. This provides a niche for the intracellular  
449 survival of *S. aureus* where it could replicate and eventually escape into the  
450 cytoplasm, ultimately leading to host cell death (24, 39). We postulated that *S.*  
451 *aureus* strain PS80 might employ a similar mechanism in BMDCs to evade  
452 killing. To assess autophagy in BMDCs, cells were infected and lysates  
453 prepared at intervals up to 6 hours post-infection and gentamicin killing of  
454 extracellular bacteria. Processing of the autophagic marker LC3 was then  
455 assessed by Western immunoblotting (40). Infection of BMDCs with *S. aureus*  
456 strain PS80 resulted in the persistence of substantial levels of LC3-II for at  
457 least 6 hours. In comparison, uninfected BMDCs or BMDCs infected with  
458 SH1000 showed no accumulation of LC3+ autophagosomes, although there  
459 was persistently a low level of LC3-II processing which was presumably due  
460 to homeostatic autophagy followed by autosome-lysosome fusion and  
461 degradation of LC3 (Figure 4A).

462

463 To confirm that PS80 was associating with autophagosomes, BMDCs were  
464 infected with CTV-labeled *S. aureus* strains PS80 or SH1000 and then treated  
465 with gentamicin to kill any extracellular bacteria. Staining with MDC (a  
466 fluorescent compound which accumulates specifically in autophagic vacuoles  
467 (41)) revealed colocalisation between PS80 and the autophagosome. SH1000  
468 was not seen to colocalise to the same extent (Figure 4B). Additionally, RAW  
469 264 macrophages that had been stably transfected with GFP-LC3 (42) were  
470 infected and gentamicin treated as above. Again, CTV-labeled PS80 was  
471 seen to colocalise with GFP-LC3 punctae at 3 hours post infection. In

472 comparison, SH1000 did not show the same level of association with GFP-  
473 LC3 punctae (Figure 4C).

474

475 To confirm that clinical isolates could also manipulate the autophagic process  
476 BMDCs were infected with Sa68 or Sa279 and lysates prepared after 6 hours.  
477 Processing of LC3 was assessed by Western immunoblotting. Similar to  
478 PS80, Sa68 infected cells had considerable levels of LC3-II present indicating  
479 a delay in the degradation of the autophagosomes. In addition, the level of  
480 LC3-II in Sa279 infected cells was similar to SH1000 infected cells or  
481 uninfected BMDCs, suggesting that these cells had normal autophagic flux  
482 (Figure 5A).

483

484 **Engagement of autophagosomes facilitates escape of *S. aureus* from**  
485 **phagocytes.**

486 To ascertain if the delay in turnover of autophagosomes was associated with  
487 the ability of *S. aureus* strains PS80 and Sa68 to escape phagocyte killing,  
488 BMDCs were pre-treated with 3-methyladenine (3-MA), a well-established  
489 PI3K inhibitor that inhibits the induction of autophagy (43), prior to infection  
490 with these two strains. The escape of *S. aureus* into the supernatant was then  
491 assessed at 6 and 12 hours. In the presence of 3-MA, PS80 and Sa68 escape  
492 into the cell culture supernatant was completely inhibited (Figure 5B).  
493 Associated with this, 3-MA treatment restored the viability of the infected  
494 BMDCs, with the level of LDH activity in the culture supernatant being  
495 significantly reduced following infection with both PS80 and Sa68 (Figure 5C).  
496 Importantly, 3-MA had no direct effect on bacterial viability after 18 hours

497 incubation ( $7.90 \pm 0.13$  vs.  $7.55 \pm 0.39$  Log CFU/ml, for *S. aureus* alone vs. *S.*  
498 *aureus* + 3-MA).

499

500 **Differential expression of Agr by *S. aureus* strains correlates with their**  
501 **ability to engage autophagosomes.**

502 It has previously been shown that the ability of *S. aureus* to divert from the  
503 endosomal pathway to autophagosomes is driven by factors that are under  
504 the control of the Agr regulatory system (24). We hypothesized that the  
505 different abilities of strains to delay autophagic flux may be associated with  
506 the level of expression of Agr. Consequently, Agr activity was measured using  
507 a vesicle lysis test (VLT). This assay measures the interaction of PSM toxins  
508 with lipid vesicles (33). The PSM $\alpha$  peptide  $\delta$ -toxin is translated from a short  
509 open reading frame located within the regulatory RNAIII molecule while  
510 transcription of the other *psm* genes is activated directly by the AgrA response  
511 regulator of the Agr two component signal transduction system that responds  
512 to high cell density. Expression of these membrane-damaging toxins is a  
513 direct manifestation of the level of expression of Agr in the stationary phase of  
514 growth (44). *S. aureus* strains PS80 and Sa68 induced significantly more  
515 vesicle lysis than SH1000 and Sa279 (Figure 6A) suggesting a greater level  
516 of Agr activity in these strains. To further assess the expression of Agr, RNAIII  
517 was measured. Consistent with the VLT, RNAIII was expressed at higher  
518 levels by *S. aureus* strains PS80 and Sa68 as compared to SH1000 and  
519 Sa279 (Figure 6B). Taken together we can conclude that the *S. aureus* strains  
520 PS80 and Sa68 that induce autophagosome accumulation, exhibit a greater

521 level of Agr activity than SH1000 and Sa279 which have no effect on  
522 autophagosomes.

523

524 **Deletion of the *agr* locus prevents LC3-II accumulation and facilitates**  
525 **bacterial killing.**

526 In order to investigate if strain dependent differences in bacterial killing and  
527 the delay of normal autophagic flux were under the control of Agr regulated  
528 genes we generated an *agr* mutant strain of PS80 by allelic exchange.  
529 BMDCs were infected with PS80 and PS80 $\Delta$ *agr* and bacterial killing was  
530 monitored over time. By 6 h post infection almost 100% of PS80 $\Delta$ *agr* were  
531 killed (Figure 7A) as compared to the parental strain, which failed to be killed.  
532 Furthermore, the escape of PS80 from the BMDCs was significantly inhibited  
533 in the absence of *agr* (1.29 $\pm$ 0.28 fold increase in Log CFU/well from T0 PS80  
534 versus 0.52 $\pm$ 0.12 fold reduction in Log CFU/well compared to T0 PS80 $\Delta$ *agr*)  
535 12 hours post infection.

536

537 In addition, the accumulation of LC3-II in infected BMDCs was also measured  
538 after 6 h of infection with PS80 or PS80 $\Delta$ *agr*. LC3-II expression was reduced  
539 in cells infected with PS80 $\Delta$ *agr* compared to the wild type, further proving that  
540 the *agr* locus plays a role in PS80's ability to block autophagic flux. However,  
541 the LC3-II processing was not reduced to baseline levels (Figure 7B),  
542 suggesting that PS80 may be expressing alternative, non-Agr regulated  
543 genes, which have some capacity to delay autophagic flux.

544

545 **Agr influences *S. aureus* persistence *in vivo*.**



546 Having established that both laboratory and clinical strains of *S. aureus* can  
547 subvert autophagy to evade phagocytic killing, it was important to determine  
548 whether this phenomenon affected infection outcome *in vivo*. Groups of wild-  
549 type mice were infected with *S. aureus* strains PS80, SH1000, Sa68, Sa279  
550 or PS80 $\Delta$ *agr* by i.p. injection. At 3 hours post challenge blood was collected  
551 and total bacterial burden in the blood was quantified (Table 2). As expected,  
552 there were significant differences in the bacterial burdens in the blood  
553 following infection with different strains. It has previously been documented  
554 that strains of *S. aureus* expressing Capsular Polysaccharide (CP) seed the  
555 blood stream from the peritoneal cavity in greater numbers than acapsular  
556 strains (45, 46). PS80 is known to express CP 8 (25), SH1000 and PS80 $\Delta$ *agr*  
557 are a-capsular (26, 47) and the CP-expression of the clinical strains are  
558 unknown. In order to prove that the differential abilities of these strains to  
559 seed the blood were not simply due to differences in CP expression levels,  
560 mice were infected with PS80 or an isogenic mutant of PS80, RMS-1 that is a-  
561 capsular (46). 3 hours post infection blood was isolated and total bacterial  
562 burden quantified. There was no significant difference in the levels of bacteria  
563 recoverable from the blood between the two groups (PS80 v RMS-1;  $4 \pm 0.2$  v  
564  $3.7 \pm 0.1$  Log CFU/ml), confirming that the observed differences in bacteremia  
565 levels were not as a result of differential CP expression.

566

567 To prove that differences in bacterial burden in the blood were due to the  
568 differential abilities of individual strains to survive intracellularly, mice were  
569 infected with *S. aureus* strains PS80, SH1000, PS80 $\Delta$ *agr*, Sa68 or Sa279. At  
570 3 hours post infection the total leukocytes were separated from the RBC and

571 extracellular bacteria by centrifugation through Histopaque 1083. Leukocytes  
572 were then washed thoroughly and lysed to quantify viable intracellular *S.*  
573 *aureus*. The number of intracellular bacteria recovered was significantly  
574 higher in PS80 infected animals compared to PS80 $\Delta$ *agr* (Figure 8A) or  
575 SH1000 (Figure 8B) infected animals. The same trend was seen in the clinical  
576 strains, with significantly higher levels of Sa68 recovered from the blood  
577 leukocytes in comparison to Sa279 (Figure 8C). This suggests that PS80 and  
578 Sa68 are capable of surviving within phagocytes *in vivo*, potentially facilitating  
579 systemic dissemination and persistence. Consistent with this, animals infected  
580 with PS80 demonstrated a significantly increased bacterial burden in the  
581 spleen at 12 hours post challenge as compared to the animals infected with  
582 the PS80 $\Delta$ *agr* (Figure 8D) or SH1000 (Figure 8E). Unfortunately, due to  
583 limitations in cell numbers we were unable to analyze autophagic flux in  
584 individual blood leukocyte populations *ex vivo*.

585

586 Finally, to establish which specific leukocyte populations in the blood were  
587 harboring intracellular *S. aureus*, GFP-expressing PS80 was injected into the  
588 peritoneum. At 3 hours post infection total leukocytes were isolated from the  
589 blood. These leukocytes were stained with a panel of antibodies against  
590 various surface markers in order to identify the phagocyte populations  
591 containing intracellular bacteria. As expected, the predominant cell type  
592 associated with GFP-expressing PS80 was found to be PMN. Surprisingly,  
593 DCs accounted for the cell type that contained the second largest population  
594 of PS80-GFP+ cells. In contrast, only a low number of monocytes were  
595 associated with PS80-GFP+ (Figure 8F). This supports the contention of this

596 study that DCs play an important direct role in phagocytosis and clearance of

597 *S. aureus*.

598

599 **Discussion**

600 Undoubtedly, the success of *S. aureus* as a pathogen can be attributed to its  
601 inherent ability to disarm the host's protective immune responses. In  
602 particular, *S. aureus* possesses a unique arsenal of virulence factors that can  
603 circumvent the bactericidal effects of phagocytes and can manipulate these  
604 cells, even parasitizing them to facilitate an intracellular lifestyle. Here we  
605 provide significant new insights into the molecular mechanisms involved.  
606 Analysis of several *S. aureus* strains revealed that, despite being  
607 phagocytosed to similar extents, some strains could elude phagocytic killing,  
608 subsequently lysing phagocytes and escaping. The ability to evade killing was  
609 directly associated with the capacity of these strains to inhibit normal  
610 autophagic flux within the cells. We showed that the ability of *S. aureus* to  
611 subvert autophagic pathways and survive within phagocytes is associated  
612 with Agr activity as strains with lower levels of Agr exhibited normal,  
613 homeostatic turnover of autophagosomes. Moreover, we established that the  
614 level of Agr expression is directly linked with the ability of *S. aureus* to survive  
615 intracellularly within phagocytes *in vivo*, suggesting that this phenomenon is  
616 related to the ability of *S. aureus* to subvert autophagy.

617

618 Previous studies have documented a protective role for dendritic cells during  
619 *S. aureus* infection. Depletion of dendritic cells was associated with increased  
620 mortality during *S. aureus* blood stream infection (36), and impaired bacterial  
621 clearance in a *S. aureus* pneumonia model (37). In both cases the beneficial  
622 effects afforded by dendritic cells were dependent upon their ability to control  
623 the inflammatory response. In this study, we demonstrated for the first time

624 that dendritic cells also have the potential to contribute to host protection by  
625 directly killing *S. aureus*. The bactericidal effects of dendritic cells were found  
626 to be comparable to those of macrophages, with both cell types being  
627 effective in reducing growth of *S. aureus* strain SH1000. Consequently  
628 we chose to use DCs as a representative phagocyte to investigate the  
629 mechanisms by which *S. aureus* can parasitize these cells. Of note, our  
630 finding contrasted with a previously published study, which concluded that  
631 BMDCs do not play a major role in direct killing of *S. aureus* (36). In that  
632 study, dendritic cells were infected with *S. aureus* at a very low ratio (MOI  
633 0.1). Given that the uptake of bacteria by macrophages has been directly  
634 linked to MOI (48), we hypothesized that bacteria must reach a critical  
635 threshold to ensure appropriate activation of the phagocyte before phagocytic  
636 killing can occur. To test this, dendritic cell killing assays were repeated using  
637 SH1000 at an MOI 0.1 and no killing was observed. However the ability of  
638 BMDCs to kill SH1000 by 16 hours became apparent at as low an MOI as 2  
639 (97.7±1.7% killing).

640

641 Our previous work demonstrated that *S. aureus* strains SH1000 and PS80  
642 possess distinct capacities to activate innate signaling pathways in dendritic  
643 cells resulting in different levels of IL-1 $\beta$  production (49). Accordingly, we  
644 wanted to dissect the interaction of these particular strains with dendritic cells.  
645 Interestingly, while both primary BMDCs and peritoneal macrophages were  
646 able to kill *S. aureus* strain SH1000 they lacked the ability to kill PS80. PS80  
647 avoided the bactericidal effects of phagocytes and instead escaped from the  
648 cells by inducing cell death. In contrast, once phagocytosed, SH1000 did not

649 escape from the phagocyte, and cells that ingested this strain remained viable  
650 for up to 24 hours post infection. Importantly both strains of *S. aureus* were  
651 efficiently phagocytosed by the dendritic cells, implying that manipulation of  
652 the phagocyte response by PS80 was exerted once it became intracellular.

653

654 *S. aureus* strain PS80 has previously been shown to survive intracellularly  
655 within neutrophils isolated from *S. aureus* surgical site infections (50). We  
656 have now demonstrated that PS80 establishes its intracellular survival niche  
657 within phagocytes through subversion of the autophagic pathway. Following  
658 infection of BMDCs, PS80 prevented the constitutive degradation of  
659 autophagosomes by lysosomes, leading to the accumulation of LC3-II. In  
660 contrast, *S. aureus* strain SH1000 did not interfere with the homeostatic  
661 turnover of the autophagic machinery. Furthermore, BMDCs that had been  
662 treated with MDC (which accumulates in the autophagosome) post-infection,  
663 showed colocalisation between the autophagosome and PS80 but not  
664 SH1000. In addition, macrophages that were stably transfected with GFP-LC3  
665 also demonstrated colocalisation of PS80 with LC3-II punctae, indicating the  
666 interaction of the bacterium with autophagosomes. Upon invasion of non-  
667 professional phagocytes *S. aureus* has been shown to subvert autophagy  
668 enabling replication within the autophagosome, and subsequent lysis of the  
669 host cell (24). Consistent with this we have demonstrated that the cytotoxic  
670 effects exerted by *S. aureus* strain PS80 on BMDCs are associated with the  
671 subversion of autophagy. Treatment of BMDCs with the autophagy inhibitor 3-  
672 MA protected cells from PS80-induced cytotoxicity and simultaneously  
673 prevented escape of the bacterium from the phagocyte.

674 Importantly, bloodstream infection isolates with comparable phenotypes to  
675 PS80 and SH1000 were identified, highlighting the clinical relevance of this  
676 phagocyte evasion strategy for facilitating systemic infection. Similar to PS80,  
677 Sa68 was not killed by BMDCs and could escape from the cells causing  
678 associated cytotoxicity. In contrast, Sa279 behaved more like SH1000 and  
679 was killed by the BMDCs. This was consistent with the observation that Sa68  
680 induced significant LC3-II accumulation in BMDCs, while inhibition of  
681 autophagy using 3-MA reduced escape of Sa68 from BMDCs.

682

683 The ability of *S. aureus* to subvert autophagy in non-phagocytic cells is  
684 controlled by the Agr system and has been shown to specifically depend upon  
685 Agr regulated expression of  $\alpha$ -toxin (Hla) (24, 39). *In vitro*, *agr* and *hla*  
686 mutants of *S. aureus* fail to trigger autophagy, are delivered efficiently to the  
687 lysosome where they are degraded and thus cannot survive intracellularly for  
688 extended periods. However, a recently published *in vivo* study has shown that  
689 while autophagy plays an important role in conferring protection against *S.*  
690 *aureus* lethality by mediating tolerance towards the cytotoxic effects of Hla,  
691 infection with a Hla mutant strain actually caused increased bacterial burden  
692 in wild-type mice in comparison to Atg16L1<sup>HM</sup> mice (that display reduced  
693 autophagy). This indicates that Hla may actually be dispensable in the  
694 exploitation of autophagy in the context of intracellular bacterial survival (51).  
695 Interestingly, when we profiled Hla expression among our strains, it did not  
696 correlate with the abilities of these strains to inhibit autophagic flux in  
697 phagocytes. *S. aureus* strains PS80 and Sa68 were comparable in their  
698 abilities to manipulate autophagy in order to evade phagocytic killing however

699 PS80 was a high Hla producer whereas Sa68 was Hla negative. Furthermore,  
700 SH1000 and Sa279 are both killed by DCs and fail to accumulate  
701 autophagosomes but SH1000 does express low levels of Hla and there is no  
702 expression detectable in Sa279 (Supplemental Fig 1). *S. aureus* strains PS80  
703 and Sa68 that evade phagocytic killing through the subversion of autophagy  
704 did express higher levels of Agr RNAIII and membrane-damaging cytolytic  
705 peptide toxins compared to SH1000 and Sa279 which did not have any  
706 appreciable effect on autophagy and were killed by the phagocytes. Crucially,  
707 we have also shown that Agr activity dictated the ability of *S. aureus* to  
708 survive within phagocytes *in vivo*. Using an *agr* mutant of PS80, we  
709 demonstrated a reduced ability of PS80 $\Delta$ *agr* to survive within leukocytes  
710 isolated from the peripheral blood following systemic challenge compared to  
711 wild-type PS80. In addition SH1000 (which exhibited reduced Agr activity)  
712 also had a significantly reduced capacity to survive within peripheral blood  
713 leukocytes *in vivo* confirming that the inability of PS80 $\Delta$ *agr* to survive in the  
714 phagocytes is not an artifact of the mutation to *agr*. Similarly, the clinical strain  
715 Sa279 (which also exhibits reduced Agr activity) shows significantly reduced  
716 survival in the circulating leukocytes in comparison to Sa68. It appears that  
717 the Agr-dependent predilection of PS80 and Sa68 for associating with  
718 autophagosomes enables them to survive within circulating leukocytes thus  
719 potentially increasing their capacity for systemic dissemination. Consistent  
720 with this, bacterial burdens in the spleen were significantly elevated in PS80  
721 infected mice as compared to animals infected with PS80 $\Delta$ *agr* or SH1000  
722 suggesting that intracellular survival in the autophagosome facilitates  
723 increased persistence in the periphery of the host.



724 Until this study Hla was the only known *S. aureus* virulence factor implicated  
725 in the induction of autophagy (39). However, the pattern of Hla expression  
726 between the strains used in this study was not sufficient to explain the  
727 phenotypes observed and it raises the question whether other Agr-regulated  
728 factors might also be capable of manipulating autophagy. Intriguingly, the VLT  
729 used to assess Agr activity measures PSM activity in culture supernatants of  
730 *S. aureus* and the pattern of vesicle lysis corresponds exactly with the  
731 observed phenotypes (33). Thus it is tempting to speculate that these toxins  
732 may also have an as yet undocumented role in the induction of autophagy in  
733 phagocytic cells. Interestingly, melittin, a component of bee sting venom that  
734 is an  $\alpha$ -helical, amphipathic antimicrobial peptide, similar to  $\delta$ -toxin (52) has  
735 previously been shown to induce autophagic cell death in trypanosomes (53).  
736 In addition  $\alpha$ PSMs trigger phagosomal escape by *S. aureus* in the monocytic  
737 cell line THP-1s (17), allowing the bacteria to replicate in the cytoplasm,  
738 leading to cell lysis (18). Autophagy has been shown to respond to both  
739 bacteria in the cytosol and within damaged phagosomes (54) supporting the  
740 notion that certain strains of *S. aureus* deliberately induce autophagy by  
741 causing damage to the phagosome. Then, by inhibiting the digestion of the  
742 autophagosomes by the lysosomes they survive within the autophagosome. A  
743 comprehensive analysis of the role played by PSMs in the induction of and  
744 engagement with autophagic pathways is warranted but is beyond the scope  
745 of this current study.

746

747 The precise mechanism by which *S. aureus* subverts autophagosomes has  
748 yet to be defined. It has previously been shown that autophagosomes may

749 form around a phagosome that has been damaged by internalized bacteria  
750 such as *Salmonella enterica* (55), suggesting that both strains of *S. aureus*  
751 may be phagocytosed normally but that PS80 may then damage the  
752 phagosome deliberately in order to secrete itself within an autophagosome.  
753 Alternatively, Gresham et al. have suggested that *S. aureus* can be taken up  
754 unconventionally by neutrophils via macropinocytosis into “large spacious  
755 vacuoles” (5). Other studies have shown that autophagy proteins can be  
756 recruited to single-membrane vacuoles such as macropinosomes (56). This  
757 may suggest an alternative internalization route for certain strains of *S.*  
758 *aureus*. While some strains are phagocytosed and killed by phagolysosomal  
759 fusion others may become internalized via macropinocytosis, which facilitates  
760 subversion of autophagic pathways in order to promote their survival.

761

762 Interestingly, PS80 can survive within several different phagocytic cell types *in*  
763 *vivo*. Consistent with previous studies (5, 50) we showed that neutrophils are  
764 the main intracellular reservoir for *S. aureus*. However DCs showed higher  
765 levels of viable intracellular bacteria than monocytes, further supporting our  
766 belief that these cells are critical in regulating the outcome of *S. aureus*  
767 infection. The primary role of DCs is to migrate to the lymph node following  
768 antigen uptake in order to activate the adaptive immune response. Therefore  
769 the ability to survive within these cells may be an attractive route of  
770 dissemination for *S. aureus*.

771

772 This study contributes to the growing literature that links subversion of  
773 autophagosomes by *S. aureus* with intracellular survival (24, 39). Our data

774 demonstrates that *S. aureus* strain PS80 and a comparable clinical isolate that  
775 express high levels of Agr prevent constitutive degradation of LC3-II+  
776 autophagosomes in order to survive and escape killing by professional  
777 phagocytes. Strains that had a lower level of Agr expression did not affect the  
778 degradation of autophagosomes in BMDCs and were efficiently killed. This  
779 study implicates autophagy as a mechanism to facilitate temporary intracellular  
780 survival of certain *S. aureus* strains within different phagocytes, maximizing  
781 their potential for dissemination and persistence *in vivo*.

782

783 The notion that *S. aureus* could parasitize neutrophils to facilitate  
784 dissemination has already been proposed (57) and our studies support the  
785 hypothesis that other phagocytes may also act as “Trojan horses” for the  
786 metastasis of *S. aureus* provided that the infecting organism possesses the  
787 appropriate tools to subvert autophagosomes. Given that our findings were  
788 replicated in clinically relevant strains, it is tempting to speculate that  
789 identifying *S. aureus* isolates which can inhibit autophagic flux by measuring  
790 Agr activity may predict invasive disease potential.

791

792 **References**

- 793 1. **Finlay BB, McFadden G.** 2006. Anti-immunology: evasion of the host  
794 immune system by bacterial and viral pathogens. *Cell* **124**:767-782.
- 795 2. **Garzoni C, Kelley WL.** 2009. Staphylococcus aureus: new evidence for  
796 intracellular persistence. *Trends Microbiol* **17**:59-65.
- 797 3. **Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B,**  
798 **Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J.**  
799 2008. A Potential New Pathway for *Staphylococcus*  
800 *aureus* Dissemination: The Silent Survival of *S.*  
801 *aureus* Phagocytosed by Human Monocyte-Derived  
802 Macrophages. *PLoS ONE* **3**:e1409.
- 803 4. **Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B,**  
804 **Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN,**  
805 **Musser JM, DeLeo FR.** 2005. Insights into mechanisms used by  
806 *Staphylococcus aureus* to avoid destruction by human neutrophils. *J*  
807 *Immunol* **175**:3907-3919.
- 808 5. **Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg**  
809 **FP.** 2000. Survival of *Staphylococcus aureus* Inside Neutrophils  
810 Contributes to Infection. *The Journal of Immunology* **164**:3713-3722.
- 811 6. **Koziel J, Maciag-Gudowska A, Mikolajczyk T, Bzowska M, Sturdevant**  
812 **DE, Whitney AR, Shaw LN, DeLeo FR, Potempa J.** 2009. Phagocytosis of  
813 *Staphylococcus aureus* by macrophages exerts cytoprotective effects  
814 manifested by the upregulation of antiapoptotic factors. *PLoS One*  
815 **4**:e5210.

- 816 7. **Kubica M, Guzik K, Koziel J, Zarebski M, Richter W, Gajkowska B,**  
817 **Golda A, Maciag-Gudowska A, Brix K, Shaw L, Foster T, Potempa J.**  
818 2008. A potential new pathway for *Staphylococcus aureus* dissemination:  
819 the silent survival of *S. aureus* phagocytosed by human monocyte-derived  
820 macrophages. *PLoS One* **3**:e1409.
- 821 8. **Schlesinger LS.** 1996. Entry of *Mycobacterium tuberculosis* into  
822 mononuclear phagocytes. *Curr Top Microbiol Immunol* **215**:71-96.
- 823 9. **Drevets DA.** 1999. Dissemination of *Listeria monocytogenes* by infected  
824 phagocytes. *Infect Immun* **67**:3512-3517.
- 825 10. **Derby BM, Rogers DE.** 1961. Studies on bacteremia. V. The effect of  
826 simultaneous leukopenia and reticuloendothelial blockade on the early  
827 blood stream clearance of staphylococci and *Escherichia coli*. *J Exp Med*  
828 **113**:1053-1066.
- 829 11. **Bera A, Herbert S, Jakob A, Vollmer W, Götz F.** 2005. Why are  
830 pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-  
831 acetyltransferase OatA is the major determinant for lysozyme resistance  
832 of *Staphylococcus aureus*. *Molecular Microbiology* **55**:778-787.
- 833 12. **Liu GY, Essex A, Buchanan JT, Datta V, Hoffman HM, Bastian JF, Fierer**  
834 **J, Nizet V.** 2005. *Staphylococcus aureus* golden pigment impairs  
835 neutrophil killing and promotes virulence through its antioxidant activity.  
836 *J Exp Med* **202**:209-215.
- 837 13. **Karavolos MH, Horsburgh MJ, Ingham E, Foster SJ.** 2003. Role and  
838 regulation of the superoxide dismutases of *Staphylococcus aureus*.  
839 *Microbiology* **149**:2749-2758.

- 840 14. **Shompole S, Henon KT, Liou LE, Dziewanowska K, Bohach GA, Bayles**  
841 **KW.** 2003. Biphasic intracellular expression of *Staphylococcus aureus*  
842 virulence factors and evidence for Agr-mediated diffusion sensing. *Mol*  
843 *Microbiol* **49**:919-927.
- 844 15. **Jarry TM, Memmi G, Cheung AL.** 2008. The expression of alpha-  
845 haemolysin is required for *Staphylococcus aureus* phagosomal escape  
846 after internalization in CFT-1 cells. *Cell Microbiol* **10**:1801-1814.
- 847 16. **Giese B, Glowinski F, Paprotka K, Dittmann S, Steiner T, Sinha B,**  
848 **Fraunholz MJ.** 2011. Expression of delta-toxin by *Staphylococcus aureus*  
849 mediates escape from phago-endosomes of human epithelial and  
850 endothelial cells in the presence of beta-toxin. *Cell Microbiol* **13**:316-329.
- 851 17. **Grosz M, Kolter J, Paprotka K, Winkler AC, Schafer D, Chatterjee SS,**  
852 **Geiger T, Wolz C, Ohlsen K, Otto M, Rudel T, Sinha B, Fraunholz M.**  
853 2014. Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal  
854 escape triggered by phenol-soluble modulins alpha. *Cell Microbiol* **16**:451-  
855 465.
- 856 18. **Surewaard BG, de Haas CJ, Vervoort F, Rigby KM, DeLeo FR, Otto M,**  
857 **van Strijp JA, Nijland R.** 2013. Staphylococcal alpha-phenol soluble  
858 modulins contribute to neutrophil lysis after phagocytosis. *Cell Microbiol*  
859 **15**:1427-1437.
- 860 19. **Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T,**  
861 **Kominami E, Ohsumi Y, Yoshimori T.** 2000. LC3, a mammalian  
862 homologue of yeast Apg8p, is localized in autophagosome membranes  
863 after processing. *EMBO J* **19**:5720-5728.

- 864 20. **Mizushima N, Yoshimori T, Levine B.** 2010. Methods in mammalian  
865 autophagy research. *Cell* **140**:313-326.
- 866 21. **Nakagawa I, Amano A, Mizushima N, Yamamoto A, Yamaguchi H,**  
867 **Kamimoto T, Nara A, Funao J, Nakata M, Tsuda K, Hamada S,**  
868 **Yoshimori T.** 2004. Autophagy defends cells against invading group A  
869 *Streptococcus*. *Science* **306**:1037-1040.
- 870 22. **Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V.**  
871 2004. Autophagy is a defense mechanism inhibiting BCG and  
872 *Mycobacterium tuberculosis* survival in infected macrophages. *Cell*  
873 **119**:753-766.
- 874 23. **Huang J, Klionsky DJ.** 2007. Autophagy and human disease. *Cell Cycle*  
875 **6**:1837-1849.
- 876 24. **Schnaith A, Kashkar H, Leggio SA, Addicks K, Kronke M, Krut O.** 2007.  
877 *Staphylococcus aureus* subvert autophagy for induction of caspase-  
878 independent host cell death. *J Biol Chem* **282**:2695-2706.
- 879 25. **Tzianabos AO, Wang JY, Lee JC.** 2001. Structural rationale for the  
880 modulation of abscess formation by *Staphylococcus aureus* capsular  
881 polysaccharides. *Proceedings of the National Academy of Sciences*  
882 **98**:9365-9370.
- 883 26. **Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ.** 2002.  
884 sigmaB modulates virulence determinant expression and stress  
885 resistance: characterization of a functional rsbU strain derived from  
886 *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**:5457-5467.
- 887 27. **Cormack BP, Valdivia RH, Falkow S.** 1996. FACS-optimized mutants of  
888 the green fluorescent protein (GFP). *Gene* **173**:33-38.

- 889 28. **Monk IR, Shah IM, Xu M, Tan MW, Foster TJ.** 2012. Transforming the  
890 untransformable: application of direct transformation to manipulate  
891 genetically *Staphylococcus aureus* and *Staphylococcus epidermidis*. *MBio*  
892 **3**.
- 893 29. **Li M, Elledge S.** 2012. SLIC: A Method for Sequence- and Ligation-  
894 Independent Cloning, p 51-59. *In* Peccoud J (ed), *Gene Synthesis*, vol 852.  
895 Humana Press.
- 896 30. **Lutz MB, Kukutsch N, Ogilvie ALJ, Rößner S, Koch F, Romani N,**  
897 **Schuler G.** 1999. An advanced culture method for generating large  
898 quantities of highly pure dendritic cells from mouse bone marrow.  
899 *Journal of Immunological Methods* **223**:77-92.
- 900 31. **Murphy AG, O'Keeffe KM, Lalor SJ, Maher BM, Mills KHG, McLoughlin**  
901 **RM.** 2014. *Staphylococcus aureus* Infection of Mice Expands a Population  
902 of Memory  $\gamma\delta$  T Cells That Are Protective against Subsequent Infection.  
903 *The Journal of Immunology* **192**:3697-3708.
- 904 32. **Harris J, Hartman M, Roche C, Zeng SG, O'Shea A, Sharp FA, Lambe**  
905 **EM, Creagh EM, Golenbock DT, Tschopp J, Kornfeld H, Fitzgerald KA,**  
906 **Lavelle EC.** 2011. Autophagy controls IL-1 $\beta$  secretion by targeting pro-  
907 IL-1 $\beta$  for degradation. *J Biol Chem* **286**:9587-9597.
- 908 33. **Laabei M, Jamieson WD, Massey RC, Jenkins AT.** 2014. *Staphylococcus*  
909 *aureus* interaction with phospholipid vesicles--a new method to  
910 accurately determine accessory gene regulator (*agr*) activity. *PLoS One*  
911 **9**:e87270.
- 912 34. **Dajcs JJ, Austin MS, Sloop GD, Moreau JM, Hume EB, Thompson HW,**  
913 **McAleese FM, Foster TJ, O'Callaghan RJ.** 2002. Corneal pathogenesis of



- 914        *Staphylococcus aureus* strain Newman. *Invest Ophthalmol Vis Sci*  
915        **43**:1109-1115.
- 916    35.    **Tan J, Lee BD, Polo-Parada L, Sengupta S.** 2012. Kinetically limited  
917        differential centrifugation as an inexpensive and readily available  
918        alternative to centrifugal elutriation. *Biotechniques* **53**:104-108.
- 919    36.    **Schindler D, Gutierrez MG, Beineke A, Rauter Y, Rohde M, Foster S,**  
920        **Goldmann O, Medina E.** 2012. Dendritic cells are central coordinators of  
921        the host immune response to *Staphylococcus aureus* bloodstream  
922        infection. *Am J Pathol* **181**:1327-1337.
- 923    37.    **Martin FJ, Parker D, Harfenist BS, Soong G, Prince A.** 2011.  
924        Participation of CD11c(+) leukocytes in methicillin-resistant  
925        *Staphylococcus aureus* clearance from the lung. *Infect Immun* **79**:1898-  
926        1904.
- 927    38.    **Baughn R, Bonventre PF.** 1975. Phagocytosis and intracellular killing of  
928        *Staphylococcus aureus* by normal mouse peritoneal macrophages. *Infect*  
929        *Immun* **12**:346-352.
- 930    39.    **Mestre MB, Fader CM, Sola C, Colombo MI.** 2010. Alpha-hemolysin is  
931        required for the activation of the autophagic pathway in *Staphylococcus*  
932        *aureus*-infected cells. *Autophagy* **6**:110-125.
- 933    40.    **Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V,**  
934        **Kaushik S, Klionsky DJ.** 2009. In search of an "autophagometer".  
935        *Autophagy* **5**:585-589.
- 936    41.    **Munafo DB, Colombo MI.** 2001. A novel assay to study autophagy:  
937        regulation of autophagosome vacuole size by amino acid deprivation. *J*  
938        *Cell Sci* **114**:3619-3629.

- 939 42. **Peral de Castro C, Jones SA, Ní Cheallaigh C, Hearnden CA, Williams L,**  
940 **Winter J, Lavelle EC, Mills KHG, Harris J.** 2012. Autophagy Regulates IL-  
941 23 Secretion and Innate T Cell Responses through Effects on IL-1  
942 Secretion. *The Journal of Immunology* **189**:4144-4153.
- 943 43. **Petiot A, Ogier-Denis E, Blommaert EF, Meijer AJ, Codogno P.** 2000.  
944 Distinct classes of phosphatidylinositol 3'-kinases are involved in  
945 signaling pathways that control macroautophagy in HT-29 cells. *J Biol*  
946 *Chem* **275**:992-998.
- 947 44. **Otto M.** 2014. Phenol-soluble modulins. *International Journal of Medical*  
948 *Microbiology* **304**:164-169.
- 949 45. **Thakker M, Park JS, Carey V, Lee JC.** 1998. *Staphylococcus aureus*  
950 serotype 5 capsular polysaccharide is antiphagocytic and enhances  
951 bacterial virulence in a murine bacteremia model. *Infect Immun* **66**:5183-  
952 5189.
- 953 46. **Watts A, Ke D, Wang Q, Pillay A, Nicholson-Weller A, Lee JC.** 2005.  
954 *Staphylococcus aureus* strains that express serotype 5 or serotype 8  
955 capsular polysaccharides differ in virulence. *Infect Immun* **73**:3502-3511.
- 956 47. **Luong T, Sau S, Gomez M, Lee JC, Lee CY.** 2002. Regulation of  
957 *Staphylococcus aureus* capsular polysaccharide expression by agr and  
958 sarA. *Infect Immun* **70**:444-450.
- 959 48. **Gog JR, Murcia A, Osterman N, Restif O, McKinley TJ, Sheppard M,**  
960 **Achouri S, Wei B, Mastroeni P, Wood JL, Maskell DJ, Cicuta P, Bryant**  
961 **CE.** 2012. Dynamics of *Salmonella* infection of macrophages at the single  
962 cell level. *J R Soc Interface* **9**:2696-2707.

- 963 49. **Maher BM, Mulcahy ME, Murphy AG, Wilk M, O'Keeffe KM, Geoghegan**  
964 **JA, Lavelle EC, McLoughlin RM.** 2013. Nlrp-3-driven interleukin 17  
965 production by gammadeltaT cells controls infection outcomes during  
966 *Staphylococcus aureus* surgical site infection. *Infect Immun* **81**:4478-  
967 4489.
- 968 50. **McLoughlin RM, Lee JC, Kasper DL, Tzianabos AO.** 2008. IFN-gamma  
969 regulated chemokine production determines the outcome of  
970 *Staphylococcus aureus* infection. *J Immunol* **181**:1323-1332.
- 971 51. **Maurer K, Reyes-Robles T, Alonzo F, 3rd, Durbin J, Torres VJ, Cadwell**  
972 **K.** 2015. Autophagy Mediates Tolerance to *Staphylococcus aureus* Alpha-  
973 Toxin. *Cell Host Microbe* **17**:429-440.
- 974 52. **Verdon J, Girardin N, Lacombe C, Berjeaud J-M, Héchard Y.** 2009.  $\delta$ -  
975 hemolysin, an update on a membrane-interacting peptide. *Peptides*  
976 **30**:817-823.
- 977 53. **Adade CM, Oliveira IRS, Pais JAR, Souto-Padrón T.** 2013. Melittin  
978 peptide kills *Trypanosoma cruzi* parasites by inducing different cell death  
979 pathways. *Toxicon* **69**:227-239.
- 980 54. **Huang J, Brumell JH.** 2014. Bacteria-autophagy interplay: a battle for  
981 survival. *Nat Rev Micro* **12**:101-114.
- 982 55. **Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH.**  
983 2006. Autophagy Controls *Salmonella* Infection in Response to Damage to  
984 the *Salmonella*-containing Vacuole. *Journal of Biological Chemistry*  
985 **281**:11374-11383.

- 986 56. **Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M.** 2011.  
987 Autophagy machinery mediates macroendocytic processing and entotic  
988 cell death by targeting single membranes. *Nat Cell Biol* **13**:1335-1343.
- 989 57. **Thwaites GE, Gant V.** 2011. Are bloodstream leukocytes Trojan Horses  
990 for the metastasis of *Staphylococcus aureus*? *Nat Rev Microbiol* **9**:215-  
991 222.  
992  
993

994 **Acknowledgements**

995 We thank Jonathan Lambourne for the collection of *S. aureus* clinical isolates  
996 (and for lineage determination).

997

998 **Funding**

999 This project was funded by a Wellcome Trust RCDF (WT086515MA) and a  
1000 Health Research Award (HRA\_POR/2012/104) to RMcL.

1001

1002 **Table 1: Primers used in deletion of *agr* from PS80.**

1003

IM293	TATACCTCGATGATGTGCATAC
IM294	GCTGATCTAACAATCCAATCCA
agr1	CCTCACTAAAGGGAACAAAAGCTGGGTACCACTCTACTAG CAAATGTTACTC
agr2	CAAACGGTCAATTTTGTTATC
agr3	CACATCGGTTGCTAAAATCCTTAATAAGATAATAAAGTCAG TTAAC
agr4	CGACTCACTATAGGGCGAATTGGAGCTCAGGATTTTAGCA ACCGATGTG
agr OUT F	AATACATAGCACTGAGTCCAAG
agr OUT R	GGGATGCCTTTATTGGTGCAG

1004

1005

1006

1007

1008 **Table 2: Bacterial burden in the blood**

	PS80	SH1000	Sa68	Sa279	PS80 $\Delta$ agr
<b>Log CFU/ml (Mean<math>\pm</math>SEM)</b>	3.63 $\pm$ 0.12	2.93 $\pm$ 0.26	3.92 $\pm$ 0.39	2.37 $\pm$ 0.49	2.28 $\pm$ 0.29
<b>Significance compared to PS80</b>	n.s.	p<0.01 **	n.s.	p<0.001 ***	p<0.0001 ***

1009

1010 **Figure Legends**

1011 **Figure 1: Killing of *S. aureus* by dendritic cells and macrophages is**  
 1012 **strain dependent.**

1013 BMDCs were infected with PS80 or SH1000 at an MOI of 10 (A) or 100 (B).  
 1014 Alternatively, peritoneal macrophages were infected with either strain at MOI  
 1015 100 (C). % killing of bacteria was determined by comparing the total CFU in  
 1016 the presence of phagocytes to bacteria in media only. Results expressed as  
 1017 mean  $\pm$  SEM at each time point, n=3/4. \*p<0.05, \*\*\*p<0.001 compared to  
 1018 other strain by repeated measures two-way ANOVA with Bonferroni post-test.

1019

1020 **Figure 2: *S. aureus* strain PS80 but not SH1000 can escape from**  
 1021 **dendritic cells causing associated cytotoxicity.**

1022 BMDCs were infected with either CTV-labeled PS80 or SH1000 at MOI 100.  
 1023 % uptake of bacteria was measured at 30 min or 2 hours (A). Following  
 1024 infection of BMDCs with PS80 or SH1000 at MOI 100, escape of each strain

1025 into the cell culture media was assessed at 6 hours and 12 hours (B). LDH  
1026 levels were assessed in the supernatant of both infected and uninfected  
1027 BMDCs (C). Results expressed as mean $\pm$ SEM (A&B) or mean $\pm$ SD (C). n=3/4  
1028 (A&B), representative of 3 independent experiments (C), \*p<0.05, \*\*\*p<0.001  
1029 by repeated measures one or two-way ANOVA with appropriate post-test.

1030

1031 **Figure 3: Identification of clinical bloodstream isolates with the ability to**  
1032 **escape phagocytic killing.**

1033 BMDC viability was screened by PI staining 24 hours post infection with a  
1034 panel of clinical strains, identifying "PS80-like" strains (black), "SH1000-like"  
1035 strains (white) and "intermediate" strains (checked) (A). BMDCs were infected  
1036 with Sa68 or Sa279 and % killing of bacteria was determined by comparing  
1037 total CFU in the presence of phagocytes to bacteria in media only (B). BMDCs  
1038 were infected with either CTV-labeled Sa68 or Sa279 at MOI 100 and %  
1039 uptake of each strain was determined by flow cytometry at 30 min or 2 hours  
1040 post infection (C). Following infection of BMDCs with Sa68 or Sa279 at MOI  
1041 100, escape of each strain into the cell culture media was assessed at 6  
1042 hours and 12 hours (D). LDH levels were assessed in the supernatant of both  
1043 infected and uninfected BMDCs (E). Results expressed as mean $\pm$ SEM (A-D)  
1044 or mean $\pm$ SD (E). n=2/6 (A-D), representative of 3 independent experiments  
1045 (E), \*p<0.05, \*\*\*p<0.001 by repeated measures one or two-way ANOVA with  
1046 appropriate post-test.

1047

1048 **Figure 4: *S. aureus* strain PS80 inhibits normal autophagic flux in**  
1049 **phagocytes.**

1050 BMDCs were infected with *S. aureus* strains PS80 or SH1000. At indicated  
1051 time points cells were lysed and expression of LC3 analysed by Western  
1052 immunoblotting. Bands show conversion of LC3-I to LC3-II.  $\beta$ -actin was  
1053 measured as a loading control. Representative blots from n=3 independent  
1054 experiments are shown (A). At 6 hours post infection with CTV-labeled  
1055 bacteria, BMDCs were stained with MDC and fixed to be viewed under a  
1056 fluorescent microscope. Blue, bacteria; yellow, MDC; white arrows indicate  
1057 colocalisation of bacteria and LC3-II (B). At 3 hours post infection with CTV-  
1058 labeled bacteria, GFP-LC3 iBMM were fixed, permeabilised and stained for  
1059 phalloidin to be viewed under a fluorescent microscope. Blue, bacteria; green,  
1060 LC3; red, phalloidin; white arrows indicate colocalisation of bacteria and  
1061 LC3-II (C). See also enlarged images showing the extent of co-localization.

1062

1063 **Figure 5: Inhibition of autophagic flux facilitates escape of *S. aureus***  
1064 **from phagocytes.**

1065 BMDCs were infected with *S. aureus* strains PS80, SH1000, Sa68 and  
1066 Sa279. At 6 hours cells were lysed and expression of LC3 analysed by  
1067 Western immunoblotting. Bands show conversion of LC3-I to LC3-II.  $\beta$ -actin  
1068 was measured as a loading control. A representative blot is shown (A).  
1069 BMDCs were pretreated with 3-MA for 30min and infected with either PS80 or  
1070 Sa68 (MOI 100). Escape of each strain into the cell culture media was



1071 assessed at 6 hours and 12 hours (B). LDH levels were assessed in the  
1072 supernatant of 3-MA pretreated and untreated BMDCs that were infected with  
1073 either PS80 or Sa68 (C). Results expressed as mean $\pm$ SEM (A&B) or  
1074 mean $\pm$ SD (C), n=4/6 (A&B), representative of 3 independent experiments (C).  
1075 \*\*\*p<0.001 by repeated measures two-way ANOVA with Bonferroni post-test.

1076

1077 **Figure 6. *S. aureus* strains exhibit distinct levels of Agr activity as**  
1078 **assessed by VLT and RNA III gene expression.**

1079 Bacterial supernatant was incubated at 1:1 ratio with lipid vesicles and  
1080 fluorescence intensity recorded as a measure of vesicle lysis (A). RNAIII  
1081 activity was measured using quantitative RT-PCR, as a ratio of RNA III and  
1082 *gyrB* transcript number (B). Results expressed as mean $\pm$ SEM. n=3/4,  
1083 \*p<0.05, \*\*p<0.01 \*\*\*p<0.001 by one-way ANOVA with Tukey post-test.

1084

1085 **Figure 7: PS80 $\Delta$ *agr* is killed by BMDCs and leads to reduced**  
1086 **accumulation of LC3-II.**

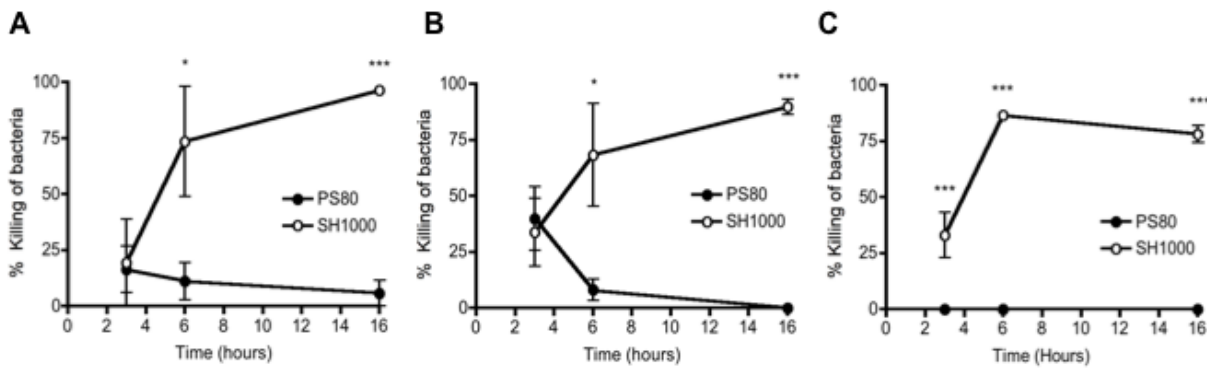
1087 BMDCs were infected with *S. aureus* strains PS80 or PS80 $\Delta$ *agr* at MOI 100.  
1088 % killing of bacteria was determined by comparing total CFU in the presence  
1089 of BMDCs to bacteria in media only (A). At 6 hours cells were lysed and  
1090 expression of LC3 analysed by Western immunoblotting.  $\beta$ -actin was  
1091 measured as a loading control. (B). Results expressed as mean  $\pm$  SEM. n=3

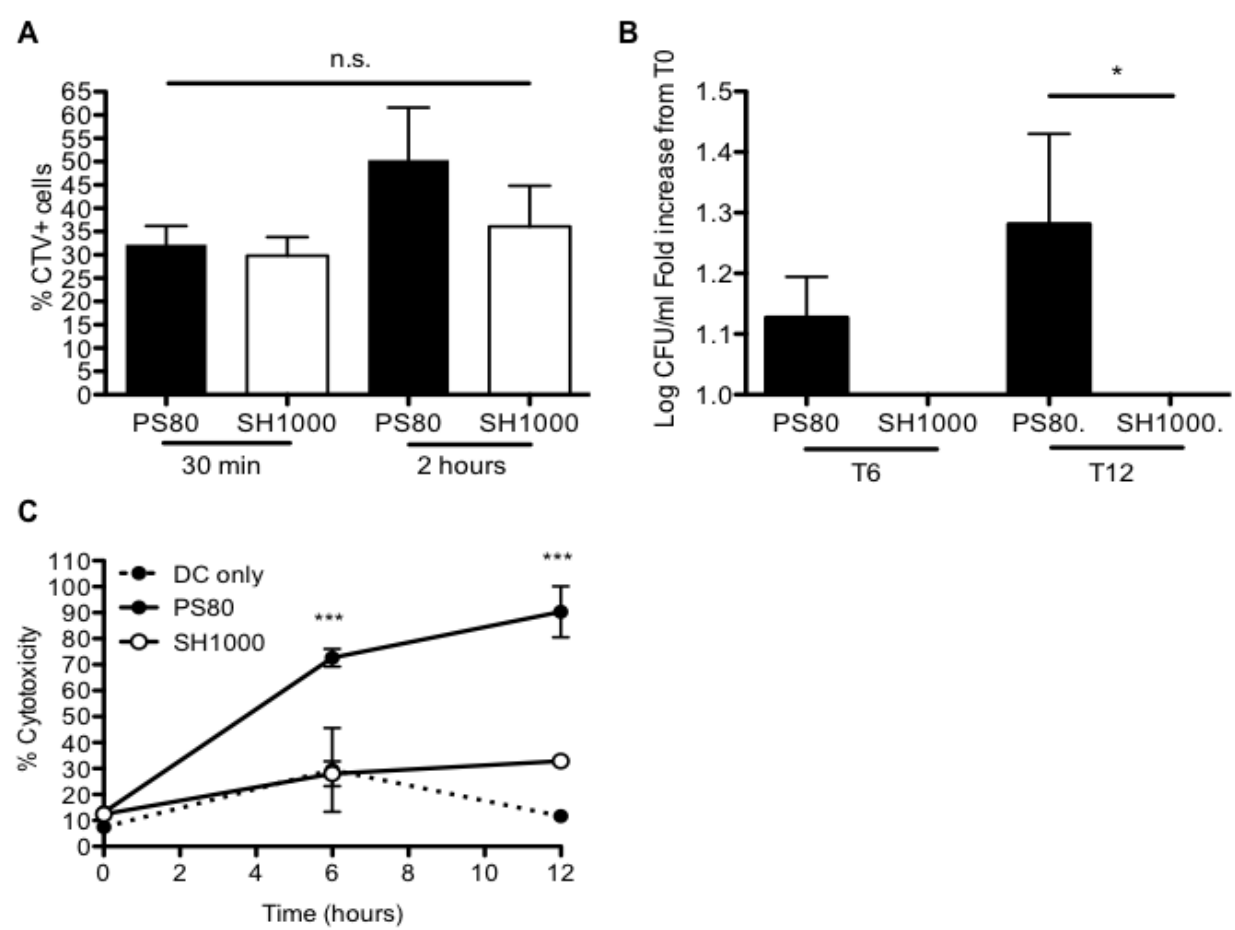
1092 (A), representative of 3 individual experiments (B), \* $p < 0.05$ , \*\* $p < 0.01$ ,  
1093 \*\*\* $p < 0.001$  by repeated measures two-way ANOVA with Bonferroni post-test.

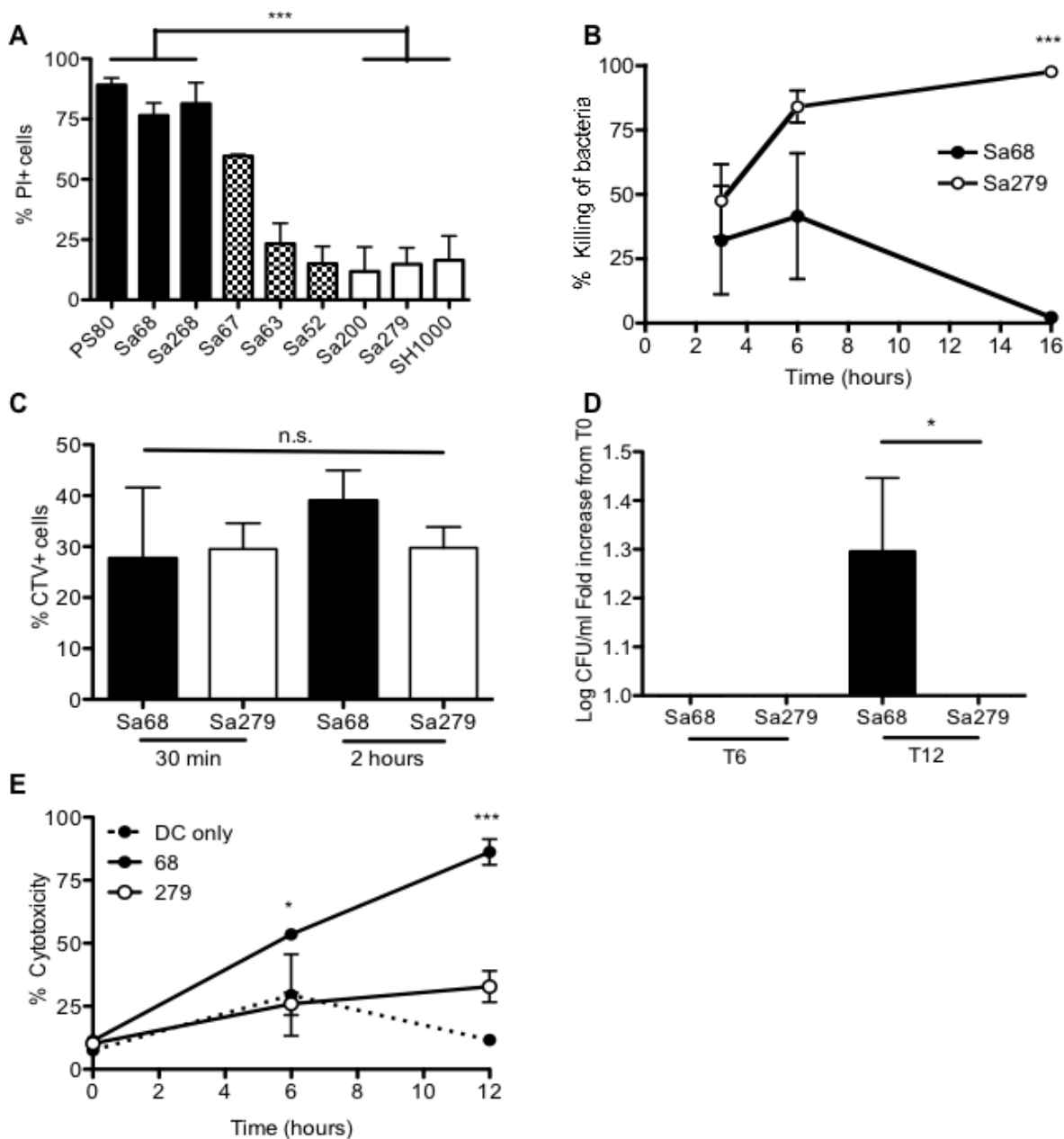
1094

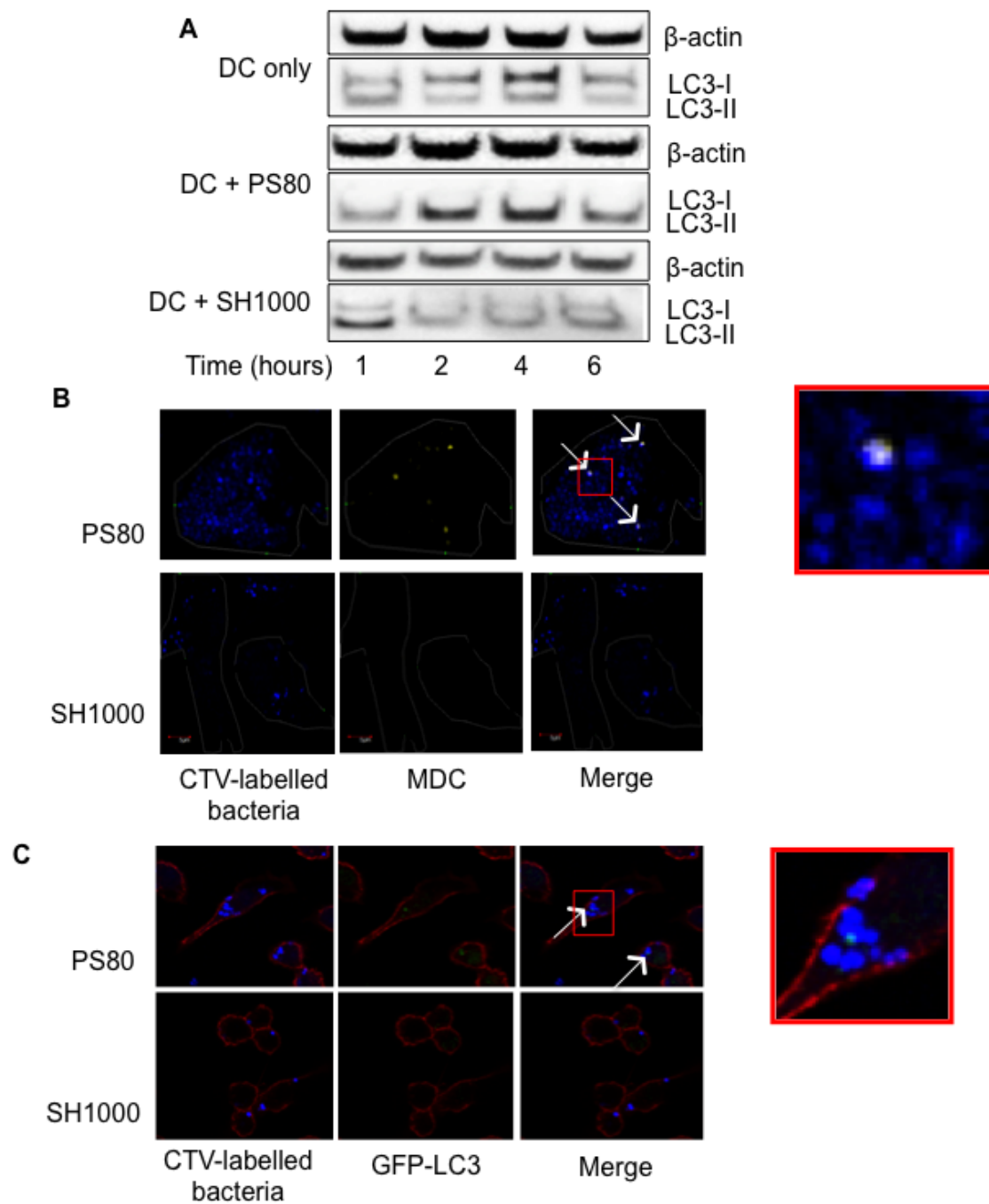
1095 **Figure 8: Intracellular persistence of *S. facilitates* infection *in vivo*.**

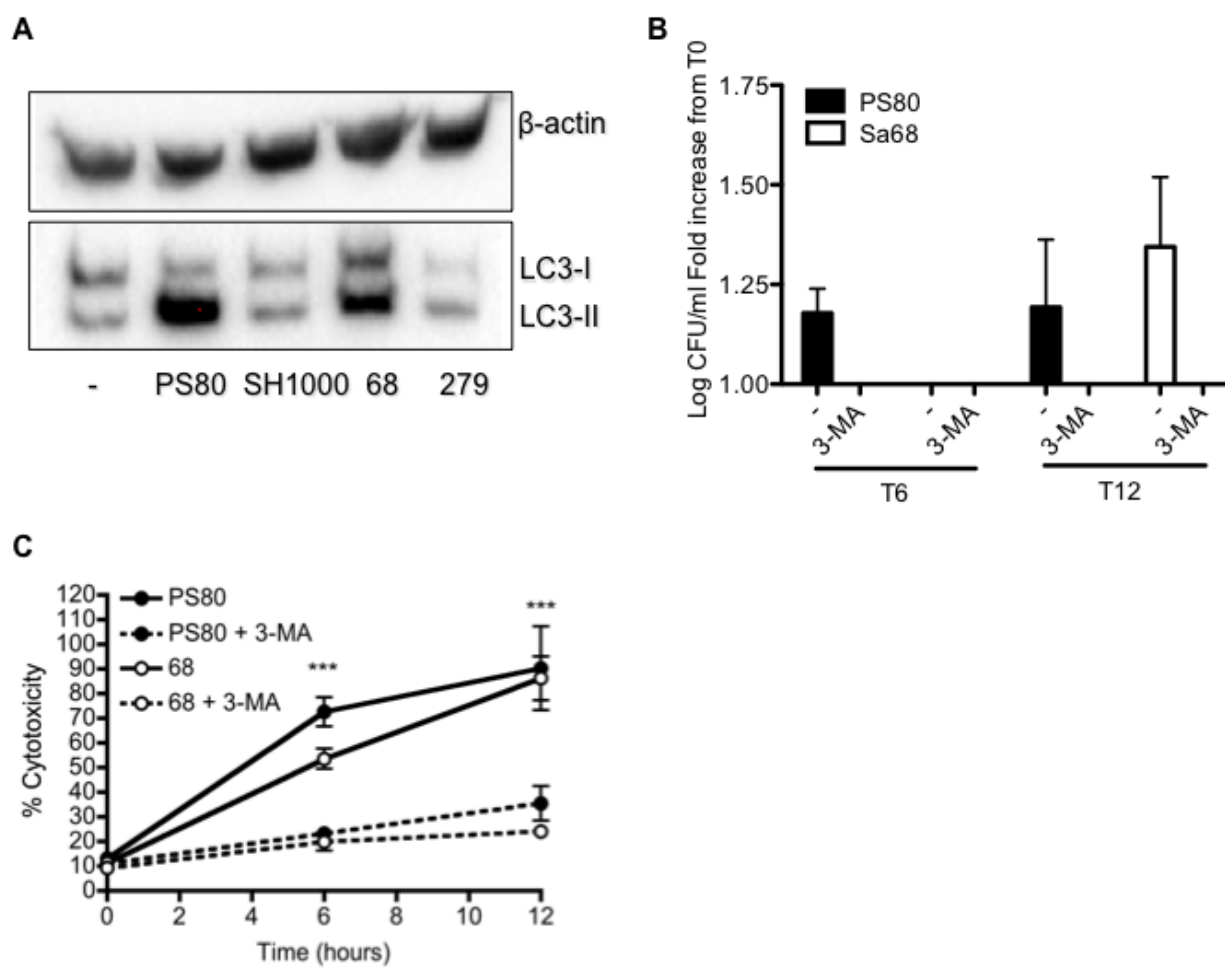
1096 Groups of mice were challenged with *S. aureus* strains PS80 (A,B,D,E),  
1097 PS80 $\Delta$ *agr* (A&D), SH1000 (B&E), Sa68, Sa279 (C) or GFP-PS80 (F) ( $5 \times 10^8$   
1098 CFU) via the intraperitoneal route. At 3 hours post challenge blood was  
1099 collected, total leukocytes isolated, washed and lysed. Cell-associated  
1100 bacteria were expressed per  $10^5$  cells (A-C). At 12 hours post challenge,  
1101 spleens were isolated, homogenized and the bacterial burden assessed  
1102 (D&E). Leukocytes isolated 3 hours post challenge were also analysed by  
1103 flow cytometry and CD11b+F480-Ly6G<sup>+</sup> (neutrophil (PMN)), CD11c<sup>+</sup>  
1104 (dendritic cells (DC)) and CD11b+F480+Ly6G<sup>-</sup> (monocyte (M $\Phi$ )) populations  
1105 that were GFP<sup>+</sup> determined (F). Results expressed as mean $\pm$ SEM, line  
1106 indicates mean,  $n=5/12$ , \* $p < 0.05$ , \*\* $p < 0.01$  by unpaired students t-test or one-  
1107 way ANOVA with Tukey post-test.

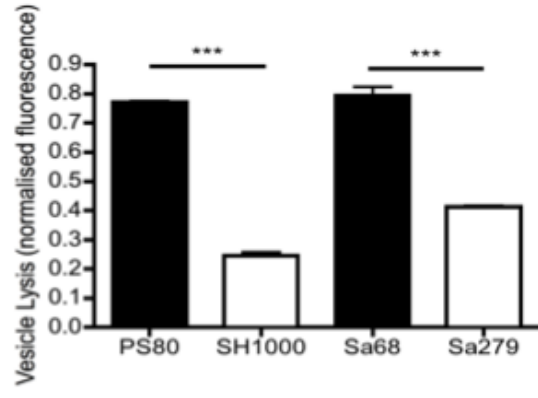










**A****B**